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Interaction of barley powdery mildew effector candidate CSEP0055 with the defence protein PR17c

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SUMMARY

A large number of effector candidates have been identified recently in powdery mildew fungi. However, their roles and how they perform their functions remain unresolved. In this study, we made use of host-induced gene silencing and confirmed that the secreted barley powdery mildew effector candidate, CSEP0055, contributes to the aggressiveness of the fungus. This result suggests that CSEP0055 is involved in the suppression of plant defence. A yeast two-hybrid screen indicated that CSEP0055 interacts with members of the barley pathogenesis-related protein families, PR1 and PR17. Interaction with PR17c was confirmed by bimolecular fluorescence complementation analyses. Down-regulation and over-expression of PR17c in epidermal cells of barley confirmed that this protein is important for penetration resistance against the powdery mildew fungus. In line with this, PR17c was found to be apoplastic, localizing to the papillae formed in response to this fungus. The *CSEP0055* transcript did not start to accumulate until 24 h after inoculation. This suggests that this gene is expressed too late to influence primary penetration events, but rather sustains the fungus at sites of secondary penetration, where PR17c appears to be able to accumulate.

INTRODUCTION

Powdery mildew fungi are obligate biotrophs, widely spread all over the world, where they afflict numerous plant species. These include the crop plants barley, wheat, grape, cucumber, tomato and many others. Powdery mildew fungi attack the plant epidermal tissue by penetrating directly through the cell wall, and subsequently form specialized organs, called haustoria, inside the plant cells, whereby nutrients are acquired from the plant. During the formation of the haustorium, a plant cell-derived membrane is generated around it, separating it from the plant cytosol.

The defence mechanisms against attack from powdery mildew fungi are mostly known from studies of barley and Arabidopsis. Initially, a cell wall-fortifying apposition, called a papilla, is formed at the site of attempted penetration (Hückelhoven and Panstruga, 2011). Papilla formation depends on a number of cell biological processes (Böhlenius *et al*., 2010; Collins *et al*., 2003; Kwon *et al*., 2008; Nielsen *et al*., 2012). In response to the pathogen, the epidermal tissue expresses many defence-related genes, including pathogenesis-related (PR) genes (Gjetting *et al*., 2007; Gregersen *et al*., 1997), and roles for some of these genes in penetration resistance have been documented (Christensen *et al*., 2004; Johrde and Schweizer, 2008). If penetration resistance fails and a haustorium develops, the attacked cell may, in incompatible interactions, execute a hypersensitive response as a programmed cell death reaction, which, in turn, also causes the biotrophic fungus to die.

In general, plants employ a broad range of molecular responses to protect themselves against microbial attack. These include the secretion of proteins to the apoplast, many of which execute hydrolytic activities on pathogen cell wall components or on proteins secreted by the pathogens. Seventeen families have been classified of the so-called PR proteins that are broadly shared between plant species (Van Loon *et al*., 2006). The molecular activities of some PR proteins, e.g. the chitinases, are well understood, whereas the functions of other families at the molecular level remain unsolved. These include the first and the last family to be classified, namely the PR1 and PR17 families.

In order to counteract the defence mechanisms of the plant, powdery mildew fungi secrete effector proteins to the plant cell. Pathogens transfer effector proteins to either the surface or interior of the host cells, where their primary purpose is to promote infection, for example by targeting and suppressing defence components (De Jonge *et al*., 2011; Dodds and Rathjen, 2010; Hogenhout *et al*., 2009; Panstruga and Dodds, 2009). For the interaction between barley and barley powdery mildew (*Blumeria graminis* f.sp. *hordei*, *Bgh*), only two effectors, AVR_{A10} and AVR_{K1}, have been described so far that suppress penetration resistance (Nowara *et al*., 2010; Ridout *et al*., 2006). AVR_{A10} and AVR_{K1} are related proteins that belong to the large EKA family of at least 1000 paralogues in *Bgh* (Ridout *et al*., 2006; Sacristán *et al*., 2009; Spanu *et al*., 2010). These proteins do not have classical signal peptides, and it remains unclear how they are transferred into the host cell, where they

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appear to interact with protein targets (Shen *et al*., 2007). Furthermore, genomic data from *Bgh* suggest that powdery mildew fungi secrete hundreds of effectors, based on classical signal peptides and default secretion (Godfrey *et al*., 2010; Spanu *et al*., 2010). These often highly expressed proteins are named *C*ANDIDATE FOR *S*ECRETED *E*FFECTOR *P*ROTEINS (CSEPs). Some of these are expected to enter the host cell. However, no entry mechanism has been described to date. In addition to having characteristic signal peptides, most of these effector candidates are small, divergent proteins unique to *Blumeria*. Almost 200 share a three-amino-acid motif, called 'YxC', in the N-terminus of the mature proteins. The first amino acid of this motif can be any of the three aromatic residues, tyrosine (Y), phenylalanine (F) or tryptophan (W), and the last is always cysteine (C) (Godfrey *et al*., 2010).

In this article, we present evidence that CSEP0055, one of these YxC-CSEPs, may be an effector. Using a yeast two-hybrid (Y2H) assay, we identified a number of PR1 and PR17 proteins interacting with this effector. Of these, we studied PR17c in more detail; it appears that this protein localizes to the papillae and makes a major contribution to penetration resistance.

RESULTS

CSEP0055 contributes to fungal success

Being one of the most highly expressed *Bgh* YxC effector candidate genes (Godfrey *et al*., 2010), we wanted to test whether *CSEP0055* (= *BghEfc3*) contributes to fungal success. An available technology for such analyses is host-induced gene silencing (HIGS), in which RNA interference (RNAi) constructs targeting fungal genes are expressed transiently in single leaf epidermal cells of barley. By an unknown mechanism, small RNAs are predicted to be transferred from the host cell to the fungus, where target transcripts are degraded (Nowara *et al*., 2010). An RNAi construct for *CSEP0055*, driven by the 35S promoter, was generated. In the *Bgh* genome, two related genes were found, but no off-targets with sufficient homology to CSEP0055 were revealed (Fig. S1, see Supporting Information). The *CSEP0055* RNAi construct was co-transformed into epidermal cells of barley with a β-glucuronidase (GUS) reporter gene construct. Two days later, the leaves were inoculated with *Bgh* and, 3 days thereafter, the leaves were stained for GUS activity. Microscopic assessment of the percentage of GUS-expressing barley epidermal cells that contained haustoria was performed, and this fungal entry rate was used as an index of aggressiveness. The data revealed that the *CSEP0055* RNAi construct reduced the fungal aggressiveness by approximately 40% relative to the empty vector control (Fig. 1). The *Mlo*-RNAi positive control construct reduced the entry rate by 75%. This reduced aggressiveness indicates that the fungal *CSEP0055* transcript is essential for *Bgh*. Furthermore, as CSEP0055 is classified as an effector candidate because of its general features, the data suggest that it may be an effector.

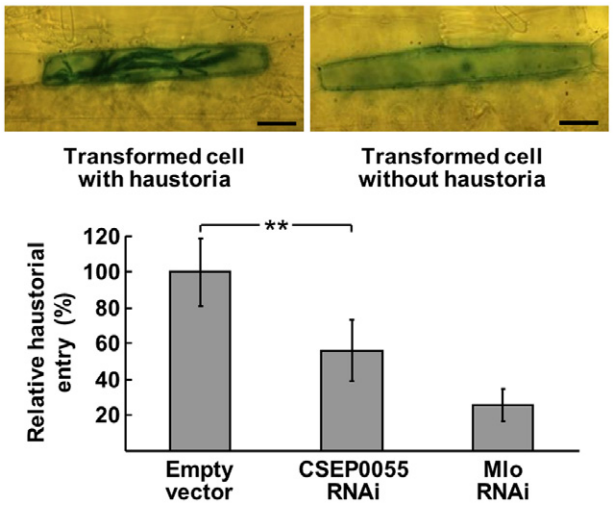

Fig. 1 Host-induced gene silencing indicates an effector role for CSEP0055. Relative haustorial entry, calculated on the basis of the number of β-glucuronidase (GUS)-expressing barley epidermal cells with and without haustoria (top), after transformation with the 35S promoter-driven *CSEP0055* RNA interference (RNAi) constructs. Data shown are mean values; $n = 5$; 100% relative entry equals 24.4% absolute entry in empty vector control; a minimum of 100 transformed cells was counted in each repeat. Error bars are ± standard deviation (SD). $^{**}P < 0.01$ (Student's *t*-test). Bars, 25 μm.

CSEP0055 interacts with PR17 and PR1 proteins

CSEP0055 has an N-terminal signal peptide (Godfrey *et al*., 2010; Spanu *et al*., 2010) predicted to be required for secretion from the fungus. It is therefore conceivable that CSEP0055 has a barley target protein, the binding of which facilitates the increased entry observed when the fungus expresses this effector (Fig. 1). In order to identify such a plant component targeted by CSEP0055, we used a Y2H assay to screen a cDNA library generated from barley leaves inoculated with *Bgh*. Using a bait plasmid expressing CSEP0055 without its signal peptide, we identified seven different full-length cDNA clones: four encoded PR17c, one encoded PR17a, one encoded PR1a and one encoded PR1b (Figs 2 and S2A, see Supporting Information). The use of another *Bgh* effector candidate, CSEP0443 (= *Bgh*Efc1) (Godfrey *et al*., 2010; Spanu *et al*., 2010), as bait for the proteins encoded by these selected clones gave no Y2H signal (Fig. S2A), supporting the specificity of the interaction of CSEP0055 with the PR proteins. Barley PR17a, PR1a and PR1b have been found previously to be apoplastic pathogen-response proteins for which the transcripts accumulate in the epidermal tissue after attack by *Bgh* (Bryngelsson *et al*., 1994; Christensen *et al*., 2002; Gregersen *et al*., 1997; Santén *et al*., 2005). However, barley PR17c has not been described to date.

As we found PR17c repeatedly by our Y2H assay, we chose to use bimolecular fluorescence complementation (BiFC) for *in vivo* confirmation of its interaction with CSEP0055 (Hu and Kerppola, 2003). This method employs two proteins of interest fused to separate

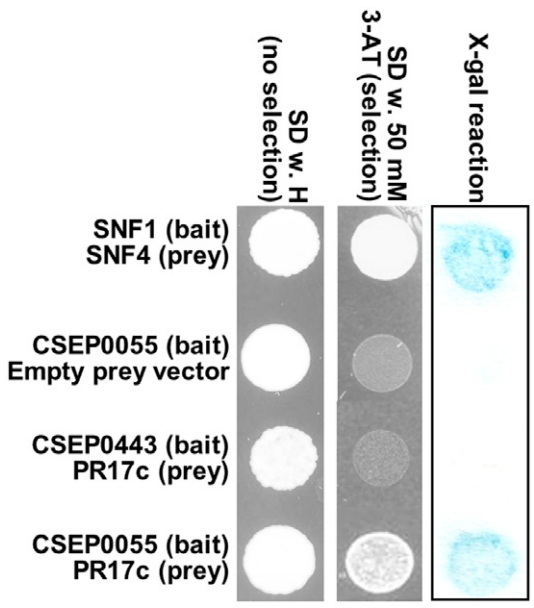

Fig. 2 Yeast two-hybrid (Y2H) identification of PR17c as an interactor of CSEP0055. Growth on solid minimal (SD) medium with 50 mM 3-amino-1,2,4-triazole (3-AT) and without histidine (H), and β-galactosidase (X-gal) activity on colony lifts made from clones grown on SD with histidine, indicate positive protein–protein interaction. SNF1/SNF4 serves as positive control. CSEP0443/PR17c serves as negative control. This experiment was repeated at least three times with similar results.

halves of a fluorescent protein. When these halves are brought in close proximity, a complete fluorophore is formed and fluorescence can be observed. Here, co-expression of 35S promoter-driven constructs expressing the fusion proteins, cCFP-PR17c and CSEP0055-nYFP (CFP, cyan fluorescent protein; YFP, yellow fluorescent protein), both without signal peptide, was obtained in tobacco leaf cells after agroinfiltration. Twenty-four hours after bacterial infiltration, the interaction was visualized using confocal laser scanning microscopy, and a strong fluorescent signal was observed (Fig. 3A). The remaining seven combinations of N- and C-terminal fusions of nYFP and cCFP with CSEP0055 and PR17c showed less signal (Table S2, see Supporting Information). As positive control, co-expression of cCFP-14-3-3 and 14-3-3-nYFP exploited the ability of 14-3-3 to form dimers (Aitken, 2006), whereas negative controls for cCFP-PR17c and CSEP0055-nYFP were obtained by co-expression of CSEP0443-nYFP/cCFP-PR17c and CSEP0055-nYFP/cCFP-14-3-3 (Figs 3B–D and S2B). This showed that the CSEP0055–PR17c interaction occurred in plants.

PR17c is an important player in barley's defence against *Bgh*

Virus-induced gene silencing in tobacco and transient over-expression in wheat suggest that PR17 homologues play roles in defence against *Pseudomonas syringae* and *B. graminis* f.sp. *tritici*, respectively (Schweizer *et al*., 1999; Xie and Goodwin, 2009). As PR17c appears to be a target for CSEP0055, we wanted to analyse its importance for barley's defence against *Bgh*. For this purpose, we used single cell transient-induced gene silencing (TIGS) to evaluate the role of individual plant genes in the interaction of single host cells with powdery mildew fungi (Douchkov *et al*., 2005). The method is performed essentially as described for the HIGS experiment (see above). Therefore, we generated a 35S promoter-driven RNAi construct that covered the 3′ part of the coding region of *PR17c* (Fig. S3, see Supporting Information). TIGS using this construct resulted in almost a tripling of the susceptibility to *Bgh* (Fig. 4A). We found that this 3′ RNAi construct has potential off-targets within the *PR17* gene family (Fig. S3). In addition to *PR17c*, it primarily targets *PR17b* and perhaps also *PR17d* and *PR17a*. Therefore, we also generated a 35S promoter-driven transcript-specific RNAi construct that covered the 5′ part of the coding region of *PR17c*. This 5′ RNAi construct almost doubled the susceptibility to *Bgh* (Fig. 4A), indicating that PR17c has a role in defence. To determine whether over-expression of PR17c could protect the plant against *Bgh*, we generated a ubiquitin promoter-driven construct with a full-length cDNA clone of *PR17c*. Transient expression in barley single cells using this construct caused a 40% decreased susceptibility to *Bgh* (Fig. 4B), which corroborated the *PR17c* RNAi results shown in Fig. 4A. Together, these combined data demonstrate the importance of PR17 proteins in defence against *Bgh*.

PR17c localizes to *Bgh*-induced papillae

The HIGS, TIGS and over-expression results shown in Figs 1 and 4 all indicated a role for PR17c in defence against *Bgh*. Therefore, we studied the localization of PR17c after attack by the powdery mildew fungus. For this purpose, a construct for ubiquitin promoter-driven expression of full-length PR17c, C-terminally fused with the red fluorescent protein mCherry (Shu *et al*., 2006), was generated. This construct was transformed into the epidermis of barley leaves by particle bombardment, together with a ubiquitin promoter-driven construct coding for CFP. In control leaves, strong nonoverlapping mCherry and CFP signals were visualized by confocal laser scanning microscopy 24 h after transformation. The CFP signal was nuclear and cytosolic, whereas the mCherry signal was present at the cell margins (Fig. 5A). That the mCherry signal was apoplastic was evident by its nonoverlapping presence outside the CFP signal (Fig. 5A; inset 1), and by its diffusion into the wall between cells neighbouring the transformed cell (Fig. 5A; inset 2). This result indicates that the PR17c signal peptide confers entry into the endoplasmic reticulum (ER), and that the fusion protein readily passes through the secretory pathway. This is in agreement with barley PR17a and PR17b being present in intercellular wash fluid (Christensen *et al*., 2002).

In order to study PR17c localization in response to *Bgh*, transformed leaves were inoculated 4 h after bombardment. Confocal

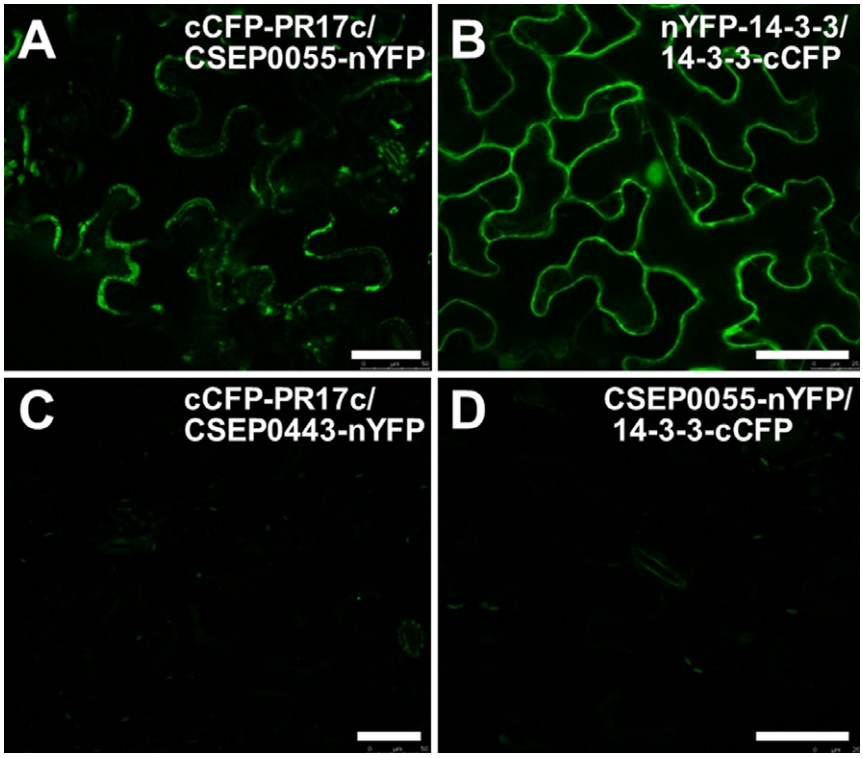

Fig. 3 CSEP0055–PR17c interaction visualized by bimolecular fluorescence complementation (BiFC) in tobacco leaves. (A) Confocal image of epidermal leaf cells from *Nicotiana benthamiana* infiltrated with a mixture of *Agrobacterium* clones harbouring T-DNA constructs for 35S promoter-driven expression of cCFP-PR17c and CSEP0055-nYFP. (B) Positive control. (C, D) Negative controls. This experiment was repeated at least three times with similar results. Bars, 50 μm.

microscopy of transformed cells 24 h after inoculation showed that PR17c-mCherry accumulated at the sites of attempted penetration as a general phenomenon (Fig. 5B). Based on the extracellular localization of the protein (Fig. 5A), this penetration site accumulation is considered to occur in the papilla. A control experiment with the transient expression of mCherry alone showed the expected cytosolic distribution and no penetration site accumulation (Fig. 5C). Determination of the emission spectrum of the red apoplastic fluorescent signal and comparison with published spectra (Wu *et al*., 2009) confirmed the presence of mCherry in the papillae and the apoplast (Fig. S4, see Supporting Information). This indicates that PR17c is responsible for papilla and apoplastic localization of the fusion protein.

CSEP0055 is involved in secondary penetration events

The HIGS and TIGS experiments determining haustorial entry above did not distinguish between primary penetrations directly from appressoria at approximately 12 h after inoculation (hai) and secondary penetrations starting at approximately 40 hai from later developing hyphae. Indeed, secondary penetrations are more numerous and therefore more prone to be affected in those experiments, all terminated at 72 hai. Transcript expression patterns could potentially be useful in determining to what extent CSEP0055 and PR17c are important for these two levels of penetration. The *PR17c* transcript was expressed from 8 hai (Caldo *et al*., 2004), suggesting that PR17c plays a role in both levels of penetration. This, however, was not the case for the *CSEP0055* transcript. In a time course experiment, little transcript was detectable until 24 hai (Fig. 6A). This makes it less likely that this effector can play a role in facilitating primary penetrations. However, from 24 hai, an increase in the *CSEP0055* transcript was found, suggesting that the HIGS effect observed in Fig. 1 may reflect a reduction in secondary penetrations.

In Fig. 5B, it is shown that PR17c has the ability to accumulate in papillae formed in response to a primary penetration event. In order to show that this protein can also accumulate in papillae at secondary penetration sites, we repeated this experiment and examined transformed cells at 48 hai. A very similar papilla accumulation was observed in multiple penetration sites (Fig. 6B). This confirms that PR17c can play a role in protecting the plant against these invasions, and that CSEP0055 may function to counteract this.

DISCUSSION

Research on powdery mildew fungi suffers from the inherent difficulty in generating transgenic lines of these organisms. Therefore, the discovery of HIGS by Nowara *et al*. (2010) provided a technology to study the roles of genes *in vivo* that was much needed to complement investigations that make use of the overexpression of fungal proteins in the plant. Here, we exploited HIGS to indicate that CSEP0055 is a significant fungal component that helps to make *Bgh* an aggressive and successful pathogen.

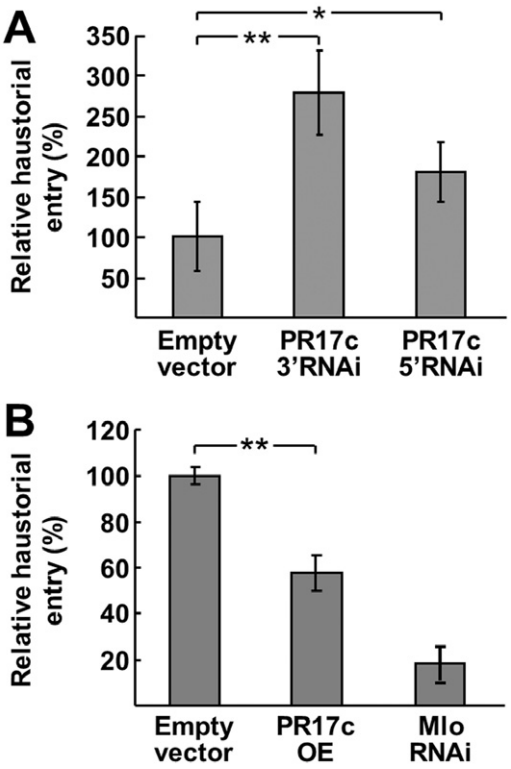

Fig. 4 Transient induced gene silencing and transient over-expression suggest that barley PR17c plays a role in defence against *Blumeria graminis* f.sp. *hordei* (*Bgh*). Relative haustorial entry, calculated on the basis of the number of β-glucuronidase (GUS)-expressing barley epidermal cells with and without haustoria (see Fig. 1) after transformation with 35S promoter-driven RNAi constructs that covered the 3′ and 5′ parts of the coding region of *PR17c* (A) and after transformation with a ubiquitin promoter-driven construct encoding full-length PR17c (B). Data shown are mean values; $n = 4$ (A) and $n = 3$ (B); 100% relative entry equals 15.8% (A) and 18.0% (B) absolute entry in empty vector controls; a minimum of 100 (A) and 200 (B) transformed cells were counted in each repeat. Error bars are ± standard deviation (SD). $^*P < 0.05$; $^{**}P < 0.01$ (Student's *t*-test).

In the Y2H screen, using CSEP0055 as bait, we identified two of each of the PR17 and PR1 barley proteins. It is well known that bacterial effectors target more than one host protein (Hann *et al*., 2010). However, keeping in mind that eukaryotic pathogens, unlike bacteria, secrete hundreds of effectors (Cantu *et al*., 2011; Jiang *et al*., 2008; Spanu *et al*., 2010), it was surprising to find more than one target for CSEP0055. Yet, Bos *et al*. (2010) identified as many as 13 potential potato targets for the *Phytophthora infestans* effector AVR3a using a Y2H assay, underlining the advanced complexity of the molecular interaction between haustoria-forming pathogens and their host plants.

Here, we chose to focus on the interaction between CSEP0055 and PR17c, which we confirmed using the BiFC method. Therefore, we analysed the role of PR17c in the defence of barley to *Bgh*. Over-expression of the wheat PR17 homologue, WCI-5, has been shown previously to cause an increased penetration resistance in wheat against *B. graminis* f.sp. *tritici* (Schweizer *et al*., 1999). Here, we performed both TIGS and over-expression of PR17c, and concluded that this protein is important for penetration resistance, as its silencing caused an almost 100% increase in *Bgh* penetration using the transcript-specific RNAi construct. In addition, PR17c over-expression reduced penetration by 40%. Strikingly, this effect of PR17c over-expression is of the same level as the effect of CSEP0055 HIGS. This suggests that the interaction between CSEP0055 and PR17c is of major importance for the outcome of the attack. TIGS using the 3′ PR17c RNAi construct, which also appears to target at least PR17b, gave a somewhat greater increased penetration. This could suggest that PR17b also plays a role in penetration resistance against *Bgh*. These levels of importance of CSEP0055 and PR17c are surprisingly high, considering that many other effectors and defence proteins must also have roles to play in the barley powdery mildew interaction. However, this might be explained by the generic functions of these proteins, as suggested below.

The discovery of two PR1 clones in our Y2H screen suggests that CSEP0055 has a more complex function as a *Bgh* effector. This result is in line with the barley data of Schultheiss *et al*. (2003), who showed the effect of TIGS using an RNAi construct for PR1b. They found an increase in the *Bgh* penetration rate by approximately 20%. It is more than 40 years since it was discovered that plants respond to pathogen attack by expressing high levels of PR1 and other PR proteins (Antoniw *et al*., 1980; Van Loon and van Kammen, 1970). However, although PR1s are the most strongly expressed PR proteins, it has been difficult to determine their biological role and molecular function. When Niderman *et al*. (1995) discovered PR1 to be antimicrobial against *Phytophthora infestans*, these proteins were believed to be specifically active against oomycetes. However, the PR1 TIGS result of Schultheiss *et al*. (2003) suggested that they also have a role in defence against fungi. In the meantime, sequence alignments have suggested that plant PR1 proteins belong to a eukaryotic CAP superfamily, some metazoan members of which have protease activity (Gibbs *et al*., 2008; Milne *et al*., 2003; Schreiber *et al*., 1997). However, no such activity has been shown for plant PR1 proteins. In the meantime, our Y2H interaction between a pathogen effector protein and PR1 proteins suggests that a new level, involving effector interactions, has been reached in the many years of PR1-associated research.

Neither PR1 nor PR17 proteins have been assigned molecular functions experimentally. However, the proteins of both families have been suggested to be proteases based on the presence of specific sequence motifs (Christensen *et al*., 2002; Gibbs *et al*., 2008; Milne *et al*., 2003). At the same time, we have seen that members of both families interact with CSEP0055 in a Y2H assay. Therefore, we attempted to make amino acid sequence alignments to search for possible sequence relationships between PR1 and

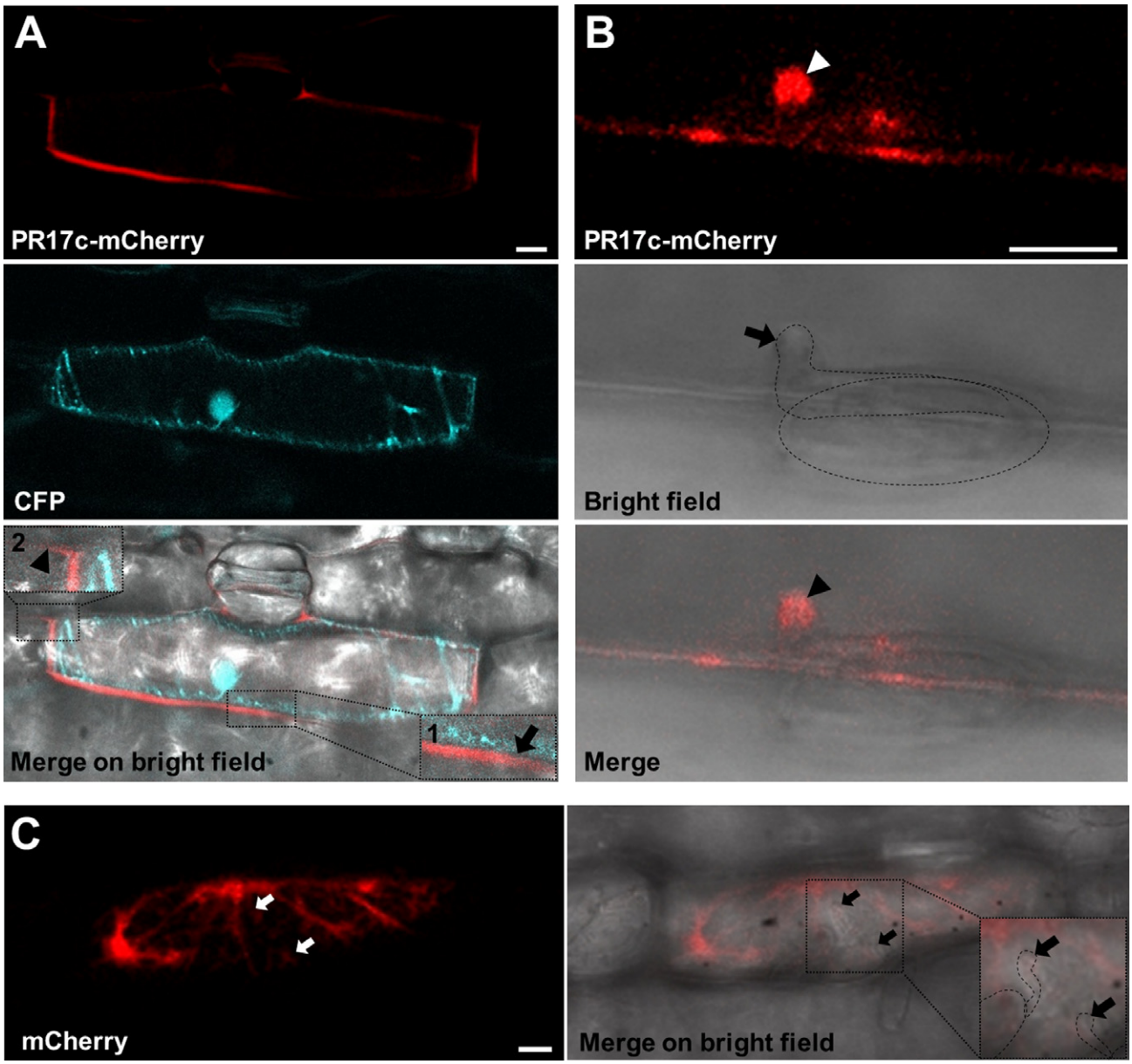

Fig. 5 PR17c localizes to *Blumeria graminis* f.sp. *hordei* (*Bgh*)-induced papillae in barley epidermal cells. (A) Confocal image of cell of noninoculated control leaf transiently transformed with constructs for ubiquitin promoter-driven expression of PR17c-mCherry and cyan fluorescent protein (CFP). The PR17c-mCherry signal is present at the cell margin and the CFP signal is cytosolic. The merged image shows that PR17c-mCherry and CFP are nonoverlapping and separated by a distance (arrow in inset 1), and that PR17c-mCherry spreads to the apoplastic space between nontransformed adjacent epidermal cells (arrowhead in inset 2). (B) Confocal image of cell transiently transformed with the PR17c-mCherry construct, recorded 24 h after inoculation. PR17c-mCherry accumulates at the site of attempted penetration (arrowhead) by a *Bgh* appressorium (arrow). (C) Confocal image of cell transiently transformed with a construct for 35S promoter-driven expression of mCherry, recorded 24 h after inoculation. mCherry is cytosolic and not focused at sites of attempted penetration (arrows). Bars, 10 μm. Stippled line silhouettes indicate spores and appressorial hyphae.

PR17. However, no such signs were encountered. Future deletion and mutant studies will show which domains of PR1 and PR17 bind to CSEP0055. Hopefully, these studies will result in a better understanding of the function of the proteins of these two families, allowing more detailed studies of the molecular consequences of CSEP0055 binding.

In this and previous studies (Christensen *et al*., 2002), we have shown that PR17 proteins are apoplastic. Furthermore, we have provided evidence that PR17c accumulates in the barley epidermal cell papillae during attack by *Bgh*. Although CSEP0055 has a signal peptide, believed to target it to the apoplast, it is conceivable that the apoplastic papilla is the meeting ground for these presumed

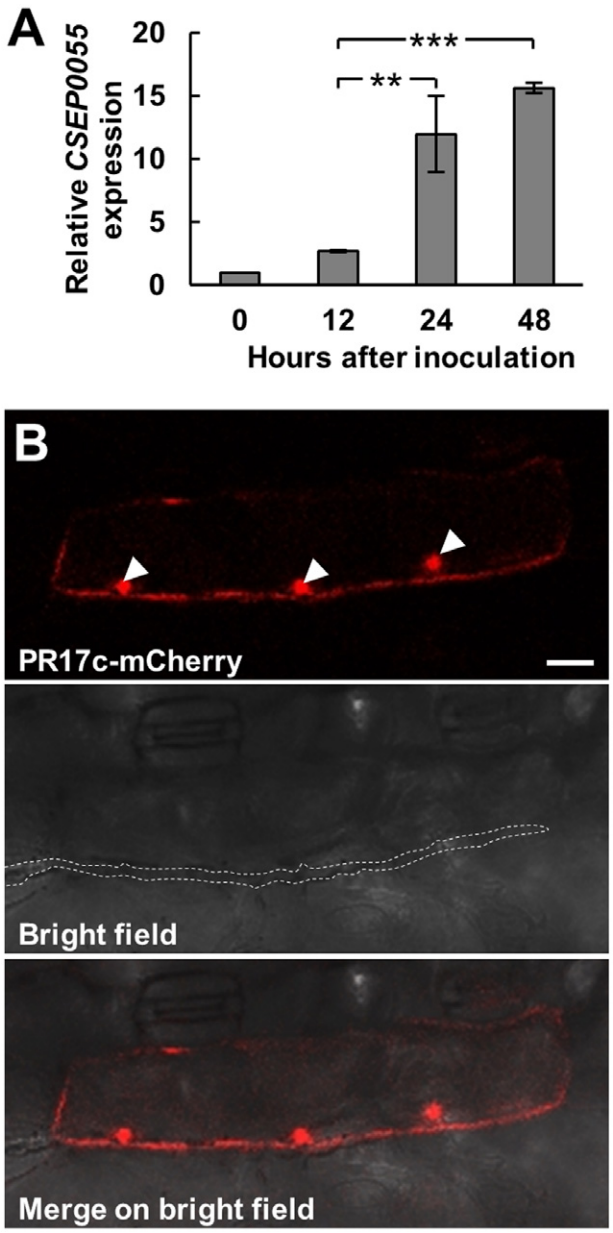

Fig. 6 *CSEP0055* expression coincides with PR17c accumulation during secondary penetration events. (A) Quantitative polymerase chain reaction (PCR) of the *CSEP0055* transcript in the fungal material on the leaf surface. The *Bgh*GAPDH transcript (GAPDH, glyceraldehyde-3-phosphate dehydrogenase) was used as reference, and the quantification was made relative to 0 h after inoculation. Each data point is based on three biological and two technical repeats. (B) Confocal image of cell transiently transformed with a construct for ubiquitin promoter-driven expression of PR17c-mCherry, recorded 48 h after inoculation. PR17c-mCherry accumulates at three sites of attempted penetration (arrowhead) from a *Bgh* secondary hypha, outlined by a stippled line silhouette. Bars, 10 μm.

important plant and pathogen disease determinants, even though the demonstration of the interaction of these proteins at this location is still awaited. Similarly, effectors from other pathogens have been demonstrated to target and inhibit apoplastic proteases and chitinases in tomato (Kaschani *et al*., 2010; Shabab *et al*., 2008; Song *et al*., 2009; Van den Burg *et al*., 2006; Van Esse *et al*., 2008). Such proteases can digest pathogen surface components. However, it would be even more biologically relevant to also digest effector proteins. Conversely, one or more fungal effectors, such as CSEP0055, could serve to inhibit these plant proteases. If this is the context in which these PR proteins function, they would not be encountered as antimicrobial in an *in vitro* assay. However, it would suggest that plants and pathogens are engaged in a battle in 'No Man's Land', exclusively aiming at destroying each other's arms. This would provide a possible explanation for the high levels of importance quantified here, in that a potential PR17c protease activity on effectors in general should have a major impact on powdery mildew aggressiveness. In turn, CSEP0055-mediated inhibition of such protease activity would be of similar importance.

EXPERIMENTAL PROCEDURES

Fungal and plant material

The barley powdery mildew fungus (*Blumeria graminis* f.sp. *hordei*, *Bgh*), isolate DH14, was maintained on susceptible barley (*Hordeum vulgare*), cv. Golden Promise, grown at 20 °C, 16 h light (150 μE/s/m^2)/8 h dark, using weekly inoculum transfer. These growth conditions were used throughout these studies. Golden Promise barley plants were also used for transient transformation and subsequent inoculation experiments. The Y2H prey cDNA library was generated from the first leaves of 11-day-old plants of P-01, a near-isogenic line of cv. Pallas with the *Bgh* resistance gene, *Mla1*, 18 hai with >100 spores/mm^2 of the virulent *Bgh* isolate, A6. BiFC studies were conducted on 2–4-week-old *Nicotiana benthamiana* plants.

Cloning procedures

For RNAi silencing the constructs of *CSEP0055* (*BghEfc3* in Godfrey *et al*., 2010) and *PR17c* (Accession number ABV22583), the sequences were polymerase chain reaction (PCR) amplified using the primer pair, EFC3_F1/EFC3_R1_stop (Table S1, see Supporting Information), on 4 ng of sscDNA from *Bgh*-infected barley epidermal material, and the primer pairs, PR17c_F3′/PR17c_R3′ and PR17c_F5′/PR17c_R5′ (Table S1), on one of the PR17c Y2H prey clones (see also Figs S1 and S3). The products were TOPO-cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA), and antisense oriented clones were selected and subsequently confirmed by sequencing. Using Gateway LR clonase reactions (Invitrogen), the inserts were transferred to the 35S promoter-driven destination vector, pIPKTA30N (Douchkov *et al*., 2005), to generate the final RNAi constructs.

For Y2H bait constructs of *CSEP0055* and *CSEP0443* (*BghEfc1* in Godfrey *et al*., 2010), the sequences were PCR amplified using the primer pairs, EFC3_F_NcoI/EFC3_R_BamHI and EFC1_F_NcoI/EFC1_R_BamHI (Table S1), on 4 ng of sscDNA from *Bgh*-infected barley epidermal. The resulting fragments were restriction digested by *Nco*I and *Bam*HI to obtain

sequence overhangs useful for cloning into the pAS2-CYH2 vector (Durfee *et al*., 1993), which was digested with the same enzyme pair. The resulting bait constructs, encoding fusions of CSEP0055 and CSEP0443 to the C-terminus of the DNA-binding domain of the GAL4 transcription factor, were confirmed by sequencing.

For the BiFC construct, CSEP0055-nYFP (nYFP denotes amino acids 1–172 of YFP), the sequence was PCR amplified using the primer pair, EFC3_F1/EFC3_R1_no-stop (Table S1), on the CSEP0055 RNAi entry clone above. For the BiFC construct, cCFP-PR17c (cCFP denotes amino acids 154–239 of CFP), the sequence was PCR amplified using the primer pair, PR17c_F1.P/PR17c_R1.P (Table S1), on one of the PR17c Y2H prey clones. For the CSEP0443-nYFP negative control construct, the sequence was PCR amplified using the primer pair, EFC1_F1/EFC1_R1_no-stop (Table S1), on the CSEP0443 bait construct above. For the BiFC control constructs, 14-3-3-cCFP and nYFP-14-3-3, a full-length coding sequence of Arabidopsis 14-3-3-lambda (At5g10450) was PCR amplified using the primer pair, oli-2134/oli-2135 (Table S1), on cDNA from Col-0. All five products were TOPO-cloned into pENTR/D-TOPO in a sense orientation based on the CACC sequence added onto the forward primers, and subsequently confirmed by sequencing. Using Gateway LR clonase reactions, the inserts were transferred to the corresponding 35S promoter-driven BiFC binary Ti vectors (Liu *et al*., 2009). These constructs were transformed into *Agrobacterium tumefaciens* (strain C58C1) and selected on Luria–Bertani (LB) plates containing 50 mg/L gentamycin, 50 mg/L spectinomycin and 10 mg/L rifampicin.

For the barley transient over-expression constructs, PR17c and PR17c-mCherry, the full-length coding sequence was PCR amplified using the primer pairs PR17c_F_SP/PR17c_R1.P and PR17c_F_SP/PR17c_R2.P separately (Table S1), on one of the PR17c Y2H prey clones. The CACC site on the forward primer ascertains directional sense-oriented TOPO cloning into pENTR/D-TOPO, which was confirmed by sequencing. Gateway LR clonase reactions were subsequently used to transfer the coding sequences into a ubiquitin promoter-driven destination over-expression vector (Shen *et al*., 2007), and a vector in which PR17c is fused to the N-terminus of the mCherry red fluorescent protein (Kwaaitaal *et al*., 2010; Shu *et al*., 2006).

A Y2H prey cDNA library was generated using polyA$^+$-RNA from the P-01 plant material, described above. The synthesized cDNA was size fractionated on a SizeSep400 spin column (Amersham Pharmacia, Amersham, UK) and ligated into the HybriZAP-2.1 Two-Hybrid Predigested Vector (Agilent Technologies, Santa Clara, CA, USA) in order to make fusions to the C-terminus of the GAL4 transcription factor activation domain. The ligated DNA was packaged with the Gigapack III Gold packaging extract (Agilent Technologies), resulting in a total of 1.75×10^6 plaque forming units. The library was amplified at this λ stage, before mass *in vivo* excision of the phagemid was performed to produce the pAD-GAL4-2.1-based prey library.

Y2H

The Y2H screen was performed as described previously (Schwechheimer and Deng, 2002) using the yeast strain Y190, containing the *HIS3* and *LacZ* reporter genes. All yeast cultures were grown in liquid or on solid minimal (SD) medium, composed of 0.7% yeast nitrogen base without amino acids (BD, Sparks, MD, USA) and 2% glucose, supplemented with adenine (A) and, depending on the transformed plasmid and Y2H interaction selection, also histidine (H), leucine (L) and tryptophan (W). All four amino acids were added to 30 μg/mL. The pAS2-CYH2-CSEP0055 bait plasmid and the barley prey cDNA library were co-transformed into the yeast strain Y190. Approximately 2×10^6 transformants were screened on solid SD with A. Furthermore, the medium included 25 mM 3-amino-1,2,4-triazole (3-AT, a competitive inhibitor of the product of the HIS3 gene). Clones exhibiting the best growth after 3 days at 30 °C were selected. To confirm Y2H interaction in the selected clones, they were grown in liquid culture (SD with A and H) to late log phase, diluted to an optical density at 600 nm (OD_{600}) of 1.0, and dropped onto solid SD with A, including 25, 50 or 75 mM 3-AT, to test the interactions after 3 days at 30 °C. A second confirmation of interaction was made by assaying the expression of the second reporter gene, *LacZ*. For this, filter-lifts of the clones grown on solid SD with A and H were incubated in an X-gal solution at 30 °C for 6–12 h to assay the yeast clones for the production of β-galactosidase, according to Bai and Elledge (1997). The double-confirmed clones were transferred to SD with A, H and W, and incubated overnight at 30 °C, to select only for the prey plasmids, which were extracted using an Easy Yeast Plasmid Isolation Kit (Clontech, Mountain View, CA, USA), and transformed into the TOP10 *Escherichia coli* strain. Sequencing was performed using designed primers for the prey vector, pAD-GAL4-2.1 (Table S1). The bait, SNF1, and the prey, SNF4, present in pAS2-CYH2 and pACT2, respectively, were used as positive controls (Durfee *et al*., 1993). CSEP0443 in pAS2-CYH2 was used as a negative bait control.

BiFC

For transient transformation by agroinfiltration of plants, the *A. tumefaciens* clones were grown overnight in LB medium with selection, harvested and resuspended in 10 mM $MgCl_2$ with 100 μM acetosyringone to OD_{600} = 0.1. The clones were mixed pairwise and incubated for 2 h at 28 °C. Using a needle-less syringe, the bacteria were infiltrated into the apoplast of leaves of 2–4-week-old *N. benthamiana* plants, and left to incubate for 24 h (Sparkes *et al*., 2006). Fluorescence in the abaxial epidermis of infiltrated leaves was analysed using a Leica SP5-X confocal laser scanning microscope with a 20× water-immersion objective with a numerical aperture of 0.7. The bimolecular fluorescence was excited at 488 nm, and the emission was detected between 518 and 540 nm, according to Hu and Kerppola (2003).

HIGS, TIGS and over-expression in barley

Silencing of genes in *Bgh* and barley was performed by barley epidermal single cell transformation after bombardment with DNA-coated gold particles, according to Nowara *et al*. (2010) and Douchkov *et al*. (2005), respectively, with adaptations described in Böhlenius *et al*. (2010). The empty vector, pIPKTA30N, was used as a negative control and the Mlo-RNAi construct, pIPKTA36, was used as a positive control (Douchkov *et al*., 2005). The existence of potential off-targets was evaluated in closely related transcripts according to the criterion that stretches of a minimum of 21 nucleotides should be shared (Brodersen and Voinnet, 2006) (see Figs S1 and S3).

Over-expression in barley epidermal single cells was obtained using the same method. For studies of protein localization in response to *Bgh* attack, the leaves were inoculated 4 h after transformation. For imaging of CFP and mCherry, a Leica SP5-X confocal laser scanning microscope was used with a 63× water-immersion lens with a numerical aperture of 1.2. CFP was excited at 458 nm and fluorescence emission was detected between 475 and 510 nm. mCherry was excited at 543 nm and fluorescence emission was detected between 580 and 620 nm. Spectral validation of the mCherry

signal was performed after excitation at 543 nm and by recording the emission between 574 and 706 nm in 12 steps within 12 -nm emission windows.

RNA isolation and quantitative (qPCR)

qPCR transcript analyses were conducted on total RNA isolated from fungal material growing exclusively on the leaf surface. This material was collected by casting it into 10% cellulose acetate (in acetone). After drying for 10 min, cellulose acetate strips detaching from the barley leaves were collected. Total RNA was isolated using the polyvinylpolypyrrolidone method (Chen *et al*., 2000). cDNA synthesis was performed using the Advantage RT-for-PCR Kit (Clontech) according to the manufacturer's instructions. Transcript quantification was performed on a Stratagene MX3000P real-time PCR detection system using the Brilliant II SYBR® Green QPCR-kit (Agilent Technologies).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 The *CSEP0055* RNA interference (RNAi) construct is transcript specific. In the *Blumeria graminis* f.sp. *hordei* (*Bgh*) genome, two *CSEP0055*-related genes were found, but none were found to be off-targets according to the criterion that stretches of a minimum of 21 nucleotides should be shared. The *CSEP0055* RNAi construct sequence is underlined.

Fig. S2 Confirmation of PR17 and PR1 proteins as interactors of CSEP0055. (A) Using CSEP0055 (without signal peptide) as bait, seven different prey cDNA clones were identified from barley (all full length): four encode PR17c, one encodes PR17a, one encodes PR1a and one encodes PR1b. Growth on solid minimal (SD) medium with 50 mM 3-amino-1,2,4-triazole (3-AT), as well as β-galactosidase (X-gal) activity on colony lifts made from clones grown on SD with histidine (H), indicates positive protein–protein interaction. SNF1/SNF4 serves as positive control. CSEP0443 serves as negative bait control. This experiment was repeated at least three times with similar results. (B) Demonstration of expression of the CSEP0443-nYFP negative bimolecular fluorescence complementation (BiFC) control. Bar, 50 μm.

Fig. S3 The 5′-*PR17c* RNA interference (RNAi) construct is transcript specific and the 3′-*PR17c* RNAi construct targets related transcripts. In the barley genome, three *PR17c*-related genes were found. None was targeted by 5′-*PR17c* RNAi (black underline), according to the criterion that stretches of a minimum of 21 nucleotides should be shared. Meanwhile, all three are potentially targeted (red underline) by 3′-*PR17c* RNAi (black underline).

Fig. S4 Emission spectra of the red fluorescent signal confirmed the presence of PR17c-mCherry in the papilla (arrowhead) and the apoplast (arrow). Blue and yellow rings, areas of signal collection. Same image as in Fig. 5B. Bar, 10 μm.

Table S1 Primer list.

Table S2 Interaction results of the eight different CSEP0055–PR17c bimolecular fluorescence complementation (BiFC) construct combinations.

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